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(54) Title: TRANSFERRIN BINDING PROTEINS FROM NEISSERIA GONORRHOEAE AND NEISSERIA MENINGITIDIS

(57) Abstract

Iron-regulated, outer membrane proteins found in *Neisseria gonorrhoeae* and *Neisseria meningitidis* are important in transferrin receptor function. The proteins, which are isolatable by means of a transferrin affinity column, bind specifically to antisera raised against an iron-regulated outer membrane protein having a molecular weight of approximately 100 kD found in *Neisseria gonorrhoeae*.

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TRANSFERRIN BINDING PROTEINS

FROM NEISSERIA GONORRHOEAE AND NEISSERIA MENINGITIDIS

This application is directed to transferrin-binding proteins from Neisseria gonorrhoeae and Neisseria meningitidis and immunologically cross-reactive fragments and analogs thereof. The specification is further directed to antibodies raised against such proteins, as well as the use of such proteins and antibodies in the detection of N. gonorrhoeae and N. meningitides and treatment of diseases caused by N. gonorrhoeae and N. meningitis. DNA encoding recombinant transferrin-binding proteins and cells that express such DNA are also covered by the present invention.

N. gonorrhoeae and N. meningitidis are two pathogens of the genus Neisseria that are genetically similar, but pathogenically different. Iron is an essential nutrient for the growth of N. gonorrhoeae and N. meningitidis, as it is for many bacteria. Unlike most other gram negative bacteria, N. gonorrhoeae and N. meningitidis do not produce and secrete small, soluble iron-chelating compounds, called siderophores. These other gram-negative bacteria have receptors capable of taking up the iron-siderophore complex.

Instead, N. gonorrhoeae and N. meningitidis are believed to possess membrane proteins that bind to the iron-binding glycoproteins lactoferrin and transferrin, which are present in human exocrine secretions and serum, respectively. N. gonorrhoeae and N. meningitidis are believed to take up iron in human hosts through the binding of lactoferrin and transferrin to these

lactoferrin- and transferrin-binding membrane proteins, i.e, receptors.

The lactoferrin-binding protein from N. meningitidis is believed to be a 105kD, iron-regulated outer membrane protein; see Schryvers and Morris, Infect. Immun. 56, 1144-1149 (1988). The transferrin-binding protein from one strain of N. meningitidis has been reported to be a 71 kD iron-regulated outer membrane protein, although other strains are reported to have transferrin-binding proteins with molecular weights of 75kD-88kD, 85kD, and 95kD; see Schryvers and Morris, Mol. Microbiol. 2, 281-288 (1988). These authors concede that the results of the various attempts at identifying the transferrin-binding protein of N. meningitidis are not consistent with each other. In fact, proteins of 85kD and 95kD were shown not to be necessary for transferrin receptor function in N. meningitidis; see Dyer et al., Microbial Pathogenesis 3, 351-363 (1987).

The ability of N. gonorrhoeae to assimilate iron has also been of interest. In one investigation, a dot binding assay involving the use of gonococcal total membranes derived from cells grown under iron-deficient conditions suggested the presence of separate receptors for lactoferrin and transferrin. The molecular weight and other properties of the binding proteins were not determined. See Lee and Schryvers, Mol. Microbiol. 2, 827-829 (1988). Therefore, the identity of the binding proteins in N. gonorrhoeae has not previously been established.

The diseases caused by gonococcal and meningococcal infection are pervasive and often serious. Improved methods for preventing, detecting and treating such

diseases, such as gonorrhea, meningitis and septic shock are needed.

The growth of N. gonorrhoeae and N. meningitidis in humans can be inhibited by reducing the ability of these cells to take up iron. A reduction in the ability of gonococcal and meningococcal cells to assimilate iron in the bloodstream could be accomplished by blocking the transferrin receptor function. The transferrin receptor, for example, could be blocked by antibodies against the receptor. In order to raise antibodies against the receptor, however, the receptor must be identified so that it can be isolated.

There is, therefore, a need for identifying, isolating and purifying the transferrin-binding proteins from N. gonorrhoeae and N. meningitidis. DNA molecules encoding such proteins are needed in order to produce recombinant transferrin binding proteins. Antibodies against the transferrin binding proteins are needed in order to inhibit transferrin receptor function. Vaccines are needed to prevent and to treat gonococcal and meningococcal infections. Antibody and nucleic acid probes are needed to detect N. gonorrhoeae and N. meningitidis. It is the object of the present invention to provide such proteins, antibodies, DNA molecules and vaccines for detecting, preventing and treating gonococcal and meningococcal infections.

SUMMARY OF THE INVENTION

These and other objectives as will become apparent to those having ordinary skill in the art have been achieved

by providing an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes,

wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein having a molecular weight of approximately 100 kD found in Neisseria gonorrhoeae; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins.

The invention further provides DNA molecules that express the transferrin binding protein and its analogs in a host cell. The resulting recombinant protein is also part of the invention.

The invention also includes antibodies against the transferrin-binding proteins of the invention. The antibodies inhibit growth of N. gonorrhoeae and/or N. meningitidis, and are useful in controlling infections of these pathogens.

The invention further includes vaccine compositions comprising the transferrin-binding proteins of the invention and analogs of such proteins, as well as methods of immunizing a host against gonococcal and meningococcal diseases, such as gonorrhea, meningitis, and septic shock, by administering such vaccines. The antibodies of the invention may be used in passive immunization to treat gonococcal and meningococcal diseases.

DESCRIPTION OF THE FIGURES

Figure 1 shows a partial DNA sequence of the 100 kD protein gene from gonococcal strain FA19 obtained from pUNCH401 and pUNCH402 inserts; see example 6a and example 7.

Figure 2A shows the sequence of a 100 kD transferrin binding protein gene fragment in a pUNCH403 insert. Capitol letters indicate the region of overlap between clones pUNCH401 and pUNCH402 (Figure 1) and pUNCH403. The proposed start codon for the 100 kD gonococcal transferrin binding protein occurs at nucleotide 659. The direction of the open reading frame is opposite that shown in the figure (i.e., from nucleotide 659 to nucleotide 1). See example 8.

Figure 3 shows positions of transposon insertions within the 100 kD gonococcal transferrin binding protein fragment in pUNCH403 and corresponding phenotypes of respective mutants. Transposons (mTn3CAT) were inserted by shuttle mutagenesis in E. coli. Chloramphenicol resistant transformants were selected in FA19 to create mutants. Below each transposon insertion (indicated by inverted triangle), growth on 2.5 uM human transferrin

(25% saturated with Fe) and expression of protein as assayed by Western blot are indicated by + or -. The open reading frame, indicated by a wavy arrow, reads right to left (same as orientation in Figure 2A) and begins with methionine, designated M. A typical -10 sequence was found (-10) but no canonical -35 sequence could be identified. Wild-type growth and protein expression are shown at right under the heading "No Tn".

Figure 4 shows the strategy for cloning the meningococcal 95 kD transferrin binding protein gene. The 1.3 kb HincII/EcoRI fragment was cloned from a lambda Zap II library using an antibody probe. The 5.0 kb and 3.5 kb fragments shown in step 2 were cloned from a partial ClaI library in pHSS6-GCU using the 1.3 kb fragment as a probe. The 3.5 kb Hinc II fragment in step 3 was cloned from a lambda Zap II library using the 3.5 kb EcoRI/ClaI fragment from step 2 as a probe. The fragments from steps 1-3 fit together as shown in the fragment entitled "FAM20 Chromosome."

Figure 5 shows the sequence of the 1.3 kb Hinc II/EcoRI fragment from step 1 of Figure 4. The ribosome binding site is underlined. The ATG start codon and direction of transcription are indicated by the arrow. See example 10.

Figure 6 shows the results of transposon mutagenesis experiments involving the 1.3 kb Hinc II/ EcoRI fragment from step 1 of Figure 4. See example 11.

DETAILED DESCRIPTION OF THE INVENTION

Isolation of Proteins from Bacteria

Transferrin-binding proteins are prepared from the membranes of N. gonorrhoeae or N. meningitidis. The membranes may be prepared by methods known in the art. The method described by Schryvers and Morris in Infect. Immun. 56, 1144-1149 (1988) is suitable. This method is incorporated herein by reference.

The membranes are obtained from cells grown in an iron-deficient medium. The growth medium may be a standard growth medium such as GC medium base (gonococcal medium base) supplied by Difco. This medium can be made iron-deficient by the addition of chelating agents such as ethylenediaminetetraacetic acid (EDTA), ethylene-diamine-di-ortho-hydroxyphenylacetic acid (EDDA), or desferal (Ciba Pharmaceuticals). Alternatively, the growth medium may be a chemically defined medium described by Mickelsen and Sparling (Inf. Immun. 33, 555-564 (1981)), which is made iron-deficient by treatment with the chelating agent Chelex-100 (Bio-Rad).

Any gonococcal and meningococcal strains that have normal transferrin receptor function are useful in the present invention. Such strains are generally available from clinical and other sources, such as the American Type Culture Collection, Bethesda, Maryland and the Neisseria Repository, NAMRU, University of California, Berkley.

For example, gonococcal strains FA19, which is described in McKenna et al, Infect. Immun. 56, 785-791 (1988); FA248, which is described in Biswas et al, J.

Bacteriol. 151, 77-82 (1979); and F62, which is described in West and Sparling, Infect. and Immun. 47, 388-394 (1985) constitute suitable sources of the gonococcal transferrin protein. Meningococcal strains FAM18 and FAM20 (Dyer et al., Microbial Pathogenesis 3, 351-363 (1987)) and B16B6, group X and group W135 (Schryvers and Morris 56, 1144-1149 (1988)) are representative of sources of the meningococcal transferrin binding protein.

Proteins that bind to transferrin may be isolated from other membrane proteins of iron-starved N. gonorrhoeae and N. meningitidis with immobilized transferrin using affinity procedures known in the art; see, for example, Schryvers and Morris, Infect. Immun. 56, 1144-1149 (1988). The method of Schryvers and Morris is incorporated herein by reference. A variation of this procedure, which is described in Example 2a, is preferably used to resolve the transferrin binding proteins from gonococcal and meningococcal membrane proteins.

Briefly, membranes from iron-starved gonococcal and meningococcal cells are isolated and treated with biotinylated transferrin. The resulting complex is immobilized by, for example, treating the complex with avidin- or streptavidin-agarose. The affinity resin pellet is thoroughly washed and suspended in buffer. The transferrin receptor is separated from the immobilized transferrin by, for example, heating. The proteins are separated by, for example, SDS-PAGE in accordance with the method of Laemmli, Nature 227, 680-685 (1970). A protein having a molecular weight of approximately 100 kD, hereinafter 100 kD protein, is resolved from gonococci. A protein having a molecular weight of approximately 95-95 kD, hereinafter, 95kD protein, is resolved from meningococci.

Identification of proteins

The molecular weights were determined by resolving single bands on SDS-PAGE and comparing their positions to those of known standards. The method is understood by those in the art to be accurate within a range of 3-5%. The molecular weights varied slightly between determinations. The molecular weight of the protein from gonococci was consistently and repeatably higher than that from meningococci, and varied from 97-100 kD.

Confirmation that the 100 kD transferrin-binding protein from N. gonorrhoeae is important for transferrin receptor function was obtained by preparing five different gonococcal mutants deficient in transferrin receptor activity. Each mutant was tested for the presence of the 100 kD transferrin-binding protein by western blot using polyclonal antisera raised in rabbits. In each mutant, the amount of 100 kD outer membrane protein was much less than was observed for wild-type gonococcal strains. Other mutant gonococcal strains that have normal transferrin receptor activity had wild-type levels of the 100 kD protein in their membranes.

A similar experiment established that the 95 kD protein from meningococci is important for transferrin receptor function. The western blot analysis was performed with antisera raised against the 100 kD protein from N. gonorrhoeae, which was found to cross-react with the 95 kD protein from N. meningitidis. Thus, in both N. gonorrhoeae and N. meningitidis, the lack of transferrin receptor activity correlates with the absence of the 100 kD and 95 kD proteins, respectively.

Therefore, contrary to expectations based on the prior art, the iron-regulated 100 kD outer membrane protein found in N. gonorrhoeae is the transferrin receptor. The iron-regulated 95 kD outer membrane protein found in N. meningitidis surprisingly cross-reacts with antisera raised against the 100 kD protein found in N. gonorrhoeae, and is the N. meningitidis transferrin receptor. Antisera raised in mammals, such as rabbits, mice, goats, monkeys and humans, against the transferrin receptor from N. gonorrhoeae are generally cross-reactive with the transferrin receptor from N. meningitidis and vice versa. Monoclonal antibodies are also generally cross-reactive with the 95 kD and 100 kD proteins.

As used herein, transferrin receptor from N. gonorrhoeae and N. meningitidis include the iron-regulated 100 kD outer membrane protein from N. gonorrhoeae and the iron-regulated 95 kD outer membrane protein from N. meningitidis. It should be understood that these transferrin receptors constitute a class of proteins. The class includes, for example, variations in the amino acid sequence that occur naturally in the various strains of N. gonorrhoeae and N. meningitidis.

The proteins of the present invention further include functional analogs of the 100 kD or the 95 kD transferrin receptors from N. gonorrhoeae or N. meningitidis, respectively. A protein is considered a functional analog of another protein for a specific function, as described below, if the analog is immunologically cross-reactive with, and has the same function as, the other protein. The analog may, for example, be a fragment of the protein, or a substitution, addition or deletion mutant of the protein.

The proteins and functional analogs of the present invention are essentially pure. For the purposes of this specification, essentially pure means that the proteins and functional analogs are free from all but trace amounts of other iron-regulated proteins from N. gonorrhoeae and N. meningitidis as well as of materials used during the purification process. The other iron-regulated proteins from N. gonorrhoeae and N. meningitidis include other transferrin binding proteins. Materials used in the purification process include detergents, affinity binding agents and separation films. Detergents include sodium dodecyl sulfate and sarcosine. Affinity binding agents include agarose, avidin-agarose, streptavidin-agarose, biotin and biotinylated proteins, such as biotinylated transferrin. Separation films include nitrocellulose paper and nitrocellulose/ cellulose acetate paper.

Recombinant DNA

Methods are known for isolating DNA once the protein has been isolated and purified. Many of these methods are described in Maniatis et al, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press (1982). The immunological screening method is preferred.

For example, chromosomal DNA from a gonococcal or meningococcal strain capable of utilizing iron bound to transferrin, such as those described above, is isolated and cleaved into fragments of suitable size by standard methods. Suitable DNA cleavage methods include, for example, sonication and the use of restriction endonucleases. A suitable average fragment size is approximately 0.5-10kbp.

Linkers are added to the fragments and the resulting fragments are ligated into a suitable vector. The linker corresponds to a restriction site in the vector. Suitable linkers include, for example, EcoRI, PstI and BamHI. A suitable vector is lambda-gt11. Ligated DNA may be packaged by commercial kits, such as a kit manufactured by Promega.

Proteins from the resulting library are cloned and expressed in a suitable host, typically E. coli. Cloning is preferably performed in an E. coli host carrying the following mutations: mcrA, mcrB, mcrC, mrr, hsdS, hsdR, and hsdM. Some suitable E. coli strains include DH5alphaMCR (BRL) and "SURE" (Stratagene).

The plaques that are obtained are screened immunologically by methods known in the art. Maniatis, Id. A suitable method is described in Example 6 below. Screening may be facilitated by the use of a commercial screening kit, such as the Picoblue Immunological Screening Kit of Stratagene (La Jolla, CA) in accordance with the accompanying Stratagene protocol, which is available from Stratagene or from the file history of this specification.

Plaques that bind the transferrin-binding protein specific antisera are selected from non-reacting plaques and purified. Maniatis, Id. The DNA from purified phage is isolated by methods known in the art. Suitable methods include, for example, polyethylene glycol precipitation, phage lysis, and anion exchange chromatography, which can be facilitated by the use of a kit manufactured by Qiagen (Studio City, CA).

The DNA obtained may be amplified by methods known in the art. One suitable method is the polymerase chain reaction (PCR) method described by Mullis et al in U.S. Patent 4,683,195 and by Sambrook, Fritch and Maniatis (eds) in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989). It is convenient to amplify the DNA clones in the lambda-gt11 vectors using lambda-gt11-specific oligomers available from New England Biolabs.

Amplified clones are inserted into suitable vectors and sequenced in accordance with methods known in the art. A suitable sequencing method is the dideoxy chain terminating method described by Sanger et al. in Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977).

Suitable vectors and polymerases for sequencing are known. A suitable vector is the Bluescript vector of Stratagene. A suitable polymerase is Sequenase (United States Biochemical Corp., Cleveland, OH).

In the immunoscreening method described above, it is usually necessary to screen a large number of plaques in order to identify fragments with the transferrin-binding protein specific antisera. For example, in one experiment, approximately 500,000 plaques were obtained from fragments of a gonococcal (FA19) chromosome. Two plaques were identified using the antisera against the 100 kD transferrin-binding protein from N. gonorrhoeae. A clone having an insert size of 323 bp (pUNCH401) was isolated from one plaque, while a clone with an insert size of 483 bp (pUNCH402) was isolated from the other plaque. These DNA sequences represent overlapping fragments of the FA19 chromosome. The consensus sequence of the two fragments, including the overlap, is shown as

Figure 1. Nucleotides 75 to 323 represent the overlapping sequences. Nucleotides 1 to 74 represent the non-overlapping sequence of the 323 bp fragment. Nucleotides 324 to 558 represent the non-overlapping sequence of the 483 bp fragment. The only open reading frame runs in the direction opposite to that shown in Figure 1 (i.e. from nucleotide 558 to nucleotide 1). See example 6a.

The fragments described above, or sub-fragments of them, can be used as probes for obtaining additional fragments of the transferrin-binding protein gene. Using this technique, an 8 kb ClaI fragment and a 3.2 kb HincII fragment in the FA19 chromosome hybridizes to the 323 and 483 bp fragments. A restriction map of the 3.2 kb HincII fragment is shown in Figure 2B. Fragments obtained can be sequenced. See examples 7 and 8 and Figure 2A.

By suitable extensions of the fragments, the entire gene is sequenced. The limits of the coding sequence are determined by methods known in the art, such as by insertional mutagenesis. See example 9. Similar methods are used to determine the sequence of the 95 kD meningococcal transferrin binding protein. See examples 10 and 11 and Figures 4-6.

Recombinant Proteins

The proteins of the present invention may be produced by means of recombinant DNA technology. Suitable methods for producing recombinant proteins from isolated DNA are described in Maniatis et al., Id.

Briefly, DNA coding for the transferrin-binding proteins of the present invention, as well as DNA coding for their functional analogs, may be expressed using a

wide variety of host cells and a wide variety of vectors. The host may be prokaryotic or eukaryotic. The DNA may be obtained from natural sources and, optionally, modified. The DNA may also be synthecized in whole or in part.

The vector may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic vectors include plasmids from E. coli such as colE1, pCR1, pBR322, pMB9, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13 and other filamentous single-stranded DNA phages.

Vectors useful in yeast are available. A suitable example is the 2u plasmid.

Suitable vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40 adenovirus, retrovirus-derived DNA sequences and vectors derived from combination of plasmids and phage DNA.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, J. Mol. Appl. Genet. 1, 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159, 601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).

Useful expression hosts include well-known prokaryotic and eukaryotic hosts. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRC1, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the transferrin-binding protein gene or fragment thereof. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

The 100 kD or 95 kD proteins may be purified by methods known in the art. For example, the entire transferrin binding proteins or portions thereof may be expressed in the form of a fusion protein with an appropriate fusion partner. The fusion partner preferably

facilitates purification and identification. Some useful fusion partners include beta-galactosidase (Gray, et al., Proc. Natl. Acad. Sci. USA 79, 6598 (1982)); trpE (Itakura et al., Science 198, 1056 (1977)) and protein A (Uhlen et al., Gene 23 369 (1983)). For example, fusion proteins containing beta-galactosidase may be purified by affinity chromatography using an anti-beta-galactosidase antibody column (Ullman, Gene. 29, 27-31 (1984)).

Optionally, the DNA that encodes the fusion protein is engineered so that the fusion protein contains a cleavable site between the transferrin binding protein and the fusion partner. Both chemical and enzymatic cleavable sites are known in the art. Suitable examples of sites that are cleavable enzymatically include sites that are specifically recognized and cleaved by collagenase (Keil et al., FEBS Letters 56, 292-296 (1975)); enterokinase (Hopp et al., Biotechnology 6, 1204-1210 (1988)); factor Xa (Nagai et al., Methods Enzymol. 153, 461-481 (1987)); thrombin (Eaton et al., Biochemistry 25, 505 (1986)); and glutathione S-transferase (Johnson, Nature 338, 585 (1989); and Van Etten et al., Cell 58, 669 (1989)). Collagenase cleaves between proline and X in the sequence Pro-X-Gly-Pro wherein X is a neutral amino acid. Enterokinase cleaves after lysine in the sequence Asp-Asp-Asp-Asp-Lys. Factor Xa cleaves after arginine in the sequence Ile-Glu-Gly-Arg. Thrombin cleaves between arginine and glycine in the sequence Arg-Gly-Ser-Pro.

Specific chemical cleavage agents are also known. For example, cyanogen bromide cleaves at methionine residues in proteins.

Alternatively, the 100 kD and 95 kD transferrin receptor proteins may be overexpressed behind an inducible

promoter and purified by affinity chromatography using specific transferrin receptor antibodies. As another alternative, the overexpressed protein may be purified using a combination of ion-exchange, size-exclusion, and hydrophobic interaction chromatography using methods known in the art. These and other suitable methods are described by Marston, "The Purification of Eukaryotic Polypeptides Expressed in E. coli" in DNA Cloning, D. M. Glover, Ed., Volume III, IRL Press Ltd., England, 1987.

UTILITY

Proteins as probes

The 100 kD protein from N. gonorrhoeae, the 95 kD protein from N. meningitidis, and their functional analogs are useful in detecting and preventing diseases caused by gonococcal and meningococcal infection.

For example, the proteins may be labelled and used as probes in standard immunoassays to detect antibodies against the proteins in samples, such as in the sera or other bodily fluids of patients being tested for gonorrhea, septic shock, or meningitis. In general, a protein in accordance with claim A or a functional derivative of such a protein is incubated with the sample suspected of containing antibodies to the protein. The protein is labelled either before, during, or after incubation. The detection of labelled protein bound to an antibody in the sample indicates the presence of the antibody. The antibody is preferably immobilized.

Suitable assays for detecting antibodies with proteins are known in the art, such as the standard ELISA protocol described by R.H. Kenneth, "Enzyme-Linked

Antibody Assay with Cells Attached to Polyvinyl Chloride Plates" in Kenneth et al, Monoclonal Antibodies, Plenum Press, N.Y., page 376 (1981). Briefly, plates are coated with a sufficient amount of an antigenic protein to bind detectable amounts of the antibody. After incubating the plates with the polypeptide, the plates are blocked with a suitable blocking agent, such as, for example, 10% normal goat serum. The sample, such as patient sera, is added and titered to determine the endpoint. Positive and negative controls are added simultaneously to quantitate the amount of relevant antibody present in the unknown samples. Following incubation, the samples are probed with goat anti-human Ig conjugated to a suitable enzyme. The presence of anti-protein antibodies in the sample is indicated by the presence of the enzyme.

For use in immunoassays, the protein may be labelled with radioactive or non-radioactive atoms and molecules. Such labels and methods for conjugating them to proteins are known in the art.

Some examples of useful radioactive labels include ^{32}P, ^{125}I, ^{131}I, and ^{3}H. Use of radioactive labels have been described in U.K. 2,034,323, U.S. 4,358,535, and U.S. 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophors, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Some useful enzymatic labels include enzymes that cause a detectable change in a substrate. Some useful enzymes and their substrates include, for example, horseradish peroxidase (pyrogallol and o-phenylene-

diamine), beta-galactosidase (fluorescein beta-D-galactopyranoside), and alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The use of enzymatic labels have been described in U.K. 2,019,404, EP 63,879, and by Rotman, Proc. Natl. Acad. Sci., 47, 1981-1991 (1961).

Useful chromophores include, for example, fluorescent, chemiluminescent, and bioluminescent molecules, as well as dyes. Some specific chromophores useful in the present invention include, for example, fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, luminol.

The labels may be conjugated to the probe by methods that are well known in the art. The labels may be directly attached through a functional group on the probe. The probe either contains or can be caused to contain such a functional group. Some examples of suitable functional groups include, for example, amino, carboxyl, sulfhydryl, maleimide, isocyanate, isothiocyanate.

The label may also be conjugated to the probe by means of a ligand attached to the probe by a method described above and a receptor for that ligand attached to the label. Any of the known ligand-receptor combinations is suitable. The biotin-avidin combination is preferred.

For use in immunoassays, the proteins may be the entire 100 kD or 95 kD protein or may be functional analogs thereof. Functional analogs of these proteins include fragments and substitution, addition and deletion mutations that do not destroy the ability of the proteins to bind to their antibodies. As long as the proteins are able to detect antibodies specific for the transferrin-

binding proteins, they are useful in the present invention.

Proteins in Vaccines

Since the transferrin-binding proteins of the present invention are important for a vital function of N. gonorrhoeae and N. meningitidis, and are found on the outer membranes, these proteins are useful in vaccines for the prevention of diseases caused by Neisseria infections, such as gonorrhea, septic shock, and meningitis. For this purpose, it is necessary for the protein to produce neutralizing antibodies. Neutralizing antibodies are antibodies that significantly inhibit the growth of and/or kill the bacterial cells in vitro or in vivo. Growth of the bacteria is significantly inhibited in vivo if the inhibition is sufficient to prevent or reduce the symptoms of the disease of a mammal infected with the disease.

Vaccines comprising the 100 kD or 95 kD protein or functional analogs as antigen may be used to inhibit the growth of, or kill, the gonococci or meningococci in accordance with the invention. Functional analogs of the 100 kD and 95 kD proteins for this purpose include fragments and substitution, addition or deletion mutations that produce neutralizing antibodies in a mammalian host such as in a human host.

The present invention further includes vaccine compositions for immunizing mammals, including humans, against infection by N. gonorrhoeae and N. meningitidis. The vaccines comprise the 100 kD transferrin receptor from N. gonorrhoeae and/or the 95 kD transferrin receptor from N. meningitidis and pharmaceutically acceptable adjuvants.

Instead of the 100 kD and 95 kD proteins, functional analogs may be substituted, as described above.

The vaccine comprises the antigen in a suitable carrier. The vaccine may include adjuvants, such as muramyl peptides, and lymphokines, such as interferon, interleukin-1 and interleukin-6. The antigen may be adsorbed on suitable particles, such as aluminum oxide particles, or encapsulated in liposomes, as is known in the art.

The antigen may also be delivered in an avirulent strain of Salmonella, such as S. typhimurium. Such vaccines may be prepared by cloning DNA comprising the active portion of the transferrin binding protein in the Salmonella strain, as is known in the art; see, for example, Curtiss et al., Vaccine 6, 155-160 (1988) and Galan et al., Gene 94, 29-35 (1990).

The invention further includes methods of immunizing host mammals, including humans, with the vaccine compositions described above. The vaccine may be administered to a mammal by methods known in the art. Such methods include, for example, intravenous, intraperitoneal, subcutaneous, or intramuscular administration.

The vaccine composition may contain the entire 100 kD protein or the 95 kD protein, but preferably contains a non-toxic fragment of the 100 kD or 95 kD protein. It is well known, for example, to produce fragments of antigenic proteins and to determine those fragments that contain the antigenic site. The length of the fragment is not critical as long as the fragment is antigenic and non-toxic. Therefore, the fragment should contain sufficient

amino acid residues to define the epitope. Methods for isolating and identifying antigenic fragments from known antigenic polypeptides are described by Salfeld et al. in J. Virol. 63, 798-808 (1989) and by Isola et al. in J. Virol. 63, 2325-2334 (1989).

If the fragment defines the epitope, but is too short to be antigenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet hemocyanin and bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

Antibodies for Treatment

Further, the invention includes isolating neutralizing antibodies that specifically recognize and bind to the proteins and functional analogs of the invention. The antibodies may be polyclonal or monoclonal. The definitions of neutralizing antibodies and functional analogs used in conjunction with vaccines (see above) apply as well to the production of neutralizing antibodies

Polyclonal antibodies are isolated from mammals that have been innoculated with the protein or a functional analog in accordance with methods known in the art. The monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method of Kohler and Milstein, Nature 256, 495-497 (1975) and the recombinant DNA method described by Huse et al. in Science 246, 1275-1281 (1989).

The invention also includes methods of treating mammals, including humans, suffering from diseases caused by N. gonorrhoeae or N. meningitidis by administering to such mammals an effective amount of the neutralizing antibodies of the invention. Administration may be by the same methods described above for administering vaccines.

Antibodies as Probes

The transferrin-binding proteins and functional analogs of the invention may also be used to produce antibodies for use as probes to detect the presence of Neisseria gonorrhoeae or Neisseria meningitidis in a sample. The antibodies may be polyclonal or monoclonal. For this purpose, functional analogs include fragments and substitution, addition and deletion mutations of the 100 kD protein or of the 95 kD protein as long as the analogs also produce antibodies capable of detecting the presence of the 100 kD or 95 kD proteins in a sample. The sample may, for example, be a bodily fluid from a mammal, including a human, suspected of being infected with N. gonorrhoeae or N. meningitidis.

Assays for detecting the presence of proteins with antibodies have been previously described, and follow known formats, such as standard blot and ELISA formats. These formats are normally based on incubating an antibody to a sample suspected of containing the 95 kD or 100 kD protein and detecting the presence of a complex between the antibody and the protein. The antibody is labelled either before, during, or after the incubation step. The protein is preferably immobilized prior to detection. Immobilization may be accomplished by directly binding the protein to a solid surface, such as a microtiter well, or by binding the protein to immobilized antibodies.

When used as probes, the antibodies are normally labelled by methods known in the art. The same labels useful for proteins (see above) are also useful for antibodies. Methods for labelling antibodies have been described, for example, by Hunter and Greenwood in Nature 144, 945 (1962) and by David et al. in Biochemistry 13, 1014-1021 (1974). Additional methods for labelling antibodies have been described in U.S. patents 3,940,475 and 3,645,090.

Nucleic Acid Molecules as Probes

Nucleic acid molecules encoding the 100 kD protein, the 95 kD protein, or fragments of the 100 kD or 95 kD proteins having unique sequences may be used to detect the presence of N. gonorrhoeae or N. meningitidis. The nucleic acid molecules may be RNA or DNA.

Methods for determining whether a nucleic acid molecule probe recognizes a specific nucleic acid molecule in a sample are known in the art. Generally, a labelled probe that is complementary to a nucleic acid sequence suspected of being in a sample is prepared. Preferably, the target nucleic acid molecule is immobilized. The presence of probe hybridized to the target nucleic acid molecule indicates the presence of the nucleic acid molecule in the sample. Examples of suitable methods are described by Dallas et al. in "The Characterization of an Escherichia Coli Plasmid Determinant that Encodes for the Production of a Heat-labile Enterotoxin." in K. N. Timmis and A. Puehler, eds, Plasmids of Medical, Environmental, and Commercial Importance, Elsevier/North-Holland Publishing Co., Amsterdam, pages 113-122 (1975); Grunstein and Hogness in Proc. Natl. Acad. Sci USA 72, 3961-3965

(1975); Palva et al. in U.S. Patent 4,731,325. which is assigned to Orion-yhtyma, Espoo, Finland; Mullis et al. in U.S. Patent 4,683,195, which is assigned to Cetus Corporation, Emeryville, California; Schneider et al. in U.S. Patent 4,882,269, which is assigned to Princeton University, and Segev in PCT Application WO 90/01069. The Schneider et al. patent and the Segev application are both licensed to ImClone Systems Inc., New York City.

The probes described above are labelled in accordance with methods known in the art. Methods for labelling oligonucleotide probes have been described, for example, by Leary et al, Proc. Natl. Acad. Sci. USA (1983) 80:4045; Renz and Kurz, Nucl. Acids Res. (1984) 12:3435; Richardson and Gumport, Nucl. Acids Res. (1983) 11:6167; Smith et al, Nucl. Acids Res. (1985) 13:2399; and Meinkoth and Wahl, Anal. Biochem. (1984) 138:267.

EXAMPLES

1. Bacterial strains and culture conditions.

Gonococcal strain FA19 is passed from frozen stock once on GCB agar and then used to inoculate flasks containing 1 liter of GCB broth to a starting density of 20 KU (Klett units). The culture is grown with 5% CO_2 at 37°C with vigorous shaking until reaching a density of 40 KU at which time the chelator, desferal, is added to a final concentration of 50 uM. Cells are harvested 4 hours after addition.

Meningococcal strain FAM20 is prepared is the same manner as gonococcal strain FA19, except for the use of Chelex-treated CDM instead of GCB and desferal.

2a. Affinity purification of gonococcal transferrin-binding protein.

The methods used for the preparation of membranes and isolation and purification of the gonococcal transferrin-binding protein is similar to that of Schryvers and Morris Infect. and Immun. 56, 1144-1149 (1988) for the preparation of meningococcal lactoferrin-binding protein. This method in the paper of Schryvers and Morris is incorporated herein by reference. The following modifications of the method of Schryvers and Morris are introduced. 625 ug of biotinylated transferrin (prepared by the method of Schryvers using Biotin-S-S-NHS from Pierce as the biotinylation reagent) is mixed with 25 mg total membrane protein from gonococcal strain FA19 in 25 ml of 100mM NaCl/50 mM Tris, pH 8.0. The mixture is incubated at room temperature for 1 hour with gentle agitation. The membranes are pelletted at 17,000 x g for 10 minutes. Pellets are resuspended in 25 ml of 100mM NaCl/50 mM Tris, pH 8.0, followed by addition of NA_2EDTA to a final concentration of 10 mM and N-lauroyl-sarcosine to a final concentration of 0.75%. Membranes are solubilized

for 10 minutes at room temperature with agitation. 2.5 ml of streptavidin-agarose (Sigma) is added and is allowed to bind for 1 hour at room temperature. The resin is spun out at 3000 x g for 5 minutes, the supernatant is removed and the resin is washed twice in 1M NaCl/50 mM Tris, pH 8.0 with 5 mM EDTA and 0.5% N-lauroyl-sarcosine and then twice in 1M NaCl/50 mM Tris, pH 8.0 with no additions. Protein is eluted from the matrix with 0.45% N-lauroyl-sarcosine and 125 mM beta-mercaptoethanol in 1M NaCl/50 mM Tris, pH 8.0.

2b. Affinity purification of meningococcal transferrin-binding protein.

The procedure of example 2a is repeated, except meningococcal strain FAM20 is substituted for gonococcal strain FA19.

3a. Isolation of gonococcal transferrin-binding protein.

The eluate from the affinity preparation (Example 2a) is concentrated using Amicon concentrators (30,000 MW cutoff). The resulting concentrated protein preparation is solubilized in 20% glycerol, 4% SDS, 130 mM Tris, pH 8.0, 10 ug/ml bromophenol blue and separated on a 7.5% SDS polyacrylamide gel according to the method of Laemmli,

Nature, 227, 680-685 (1970). The gel is stained with Coomassie Brilliant Blue to visualize the proteins. Two protein species are resolved as single bands by this method. Transferrin has a molecular weight of approximately 80kD. The transferrin-binding protein has a molecular weight of 100 kD. The 100 kD protein band is excised, lyophilized and macerated.

3b. Isolation of meningococcal transferrin-binding protein.

The procedure of example 3A is repeated, except the eluate from example 2b is substituted for the eluate of example 2a.

4. Antisera against the transferrin-binding protein.

The fine powders resulting from examples 3a and 3b are separately resuspended in saline, mixed with an equal volume of Freund's adjuvant (complete for the first injection; incomplete for subsequent injections) and injected into New England White, female rabbits. Injections are spaced two weeks apart. Anti-100 kD protein antibody can be detected two weeks after the third injection by western blotting against purified transferrin-binding protein.

5a. Gonococcal DNA lambda-gt11 expression library.

Chromosomal DNA from gonococcal strain FA19 is isolated according to Seifert et al, J. Bacteriol. 172, 40-46 (1990) and sonicated by standard procedures (Maniatis et al, 1982) to yield an average fragment size of 500 bp. EcoRI linkers are added and the resulting fragments are ligated into EcoRI digested lambda-gt11 DNA (Maniatis et al, 1982). Ligated DNA is packaged using a kit manufactured by Promega.

5b. Meningococcal DNA lambda-gt11 expression library.

Chromosomal DNA from meningococcal strain FAM20 is isolated in accordance with Seifert et al, J. Bacteriol. 172, 40-46 (1990) and digested with the restriction endonuclease HincII. EcoR1 linkers are added, and the resultant DNA molecule is digested with EcoR1 and ligated into EcoR1 digested lambda-Zap (Stratagene). Ligated DNA is packaged using a kit manufactured by Promega.

6a. Immunological screening of the expression library.

Approximately 500,000 plaques obtained from the library of examples 5a and 5b are screened by the immunological screening method described in Stratagene's

protccol accompanying the Picoblue Immunological Screening Kit. Briefly, the primary antisera is absorbed with an E. coli/phage lysate available from Stratagene (LaJolla, CA) according to their protocol. Approximately $5x10^4$ pfu (plaque forming units) are plated on the E. coli host strain, Y1090. Nitrocellulose filters, soaked in 10 mM isopropylthiogalactoside (IPTG) are laid upon plates following 3-4 hours incubation at 42°C. Plates are then incubated overnight after which filters are removed, washed in tris-buffered saline and 0.05% Tween-20 (TBST) and blocked for one hour in tris-buffered saline and 5% bovine serum albumen. The filters are then incubated with a 1:200 dilution of the absorbed primary antibody for one hour. After incubation with primary antibody, filters are washed extensively with TBST and then incubated with the secondary antibody (1:3000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase, purchased from Bio-Rad) for one hour. Filters are then washed extensively with TBST and finally incubated in 0.3 mg/ml nitroblue tetrazolium (NBT), 0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP), 100 mM Tris pH 9.8, 100 mM NaCl, 5 mM $MgCl_2$ until sufficient color develops.

Plaques which bind the transferrin-binding protein specific antisera are picked and purified away from other non-reacting plaques. The DNA from purified phage is

isolated and purified using anion-exchange chromatography (column purchased from Qiagen, Studio City, CA).

6b. Screening the expression library with DNA probes

Plagues obtained from the library of examples 5a and 5b are also screened using labeled DNA probes. Oligomer TfBP1, 2, 3, or 5 is labeled nonradioactively using digoxigenin-11-dUTP and a DNA tailing kit, both manufactured by Boehringer Mannheim Biochemicals (BMB). The sequences of the oligomers are:

TFBP1: GAG CCC GCC AAT GCG CCG CT

TFBP2: AGC GGC GCA TTG GCG GGC TC

TFBP3: GGG GCG CAT CGG CGG TGC GG

TFBP5: AAA ACA GTT GGA TAC CAT AC

The protocol for DNA labeling and detection are available from BMB with the Genius nonradioactive dna labeling and detecting kit. Alternatively, the same oligomers are labelled radioactively with alpha-32p-dCTP and BMB's DNA tailing kit using standard techniques (Maniatis et al, 1982).

7. Amplification and sequencing of DNA.

The DNA obtained in example 5 or 6 is amplified by the PCR technique (Sambrook et al, (eds), Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Press (1989)) using lambda-gt11-specific oligomers as amplimers. Inserts thus amplified are cloned into Bluescript vectors (Stratagene) using standard techniques (Maniatis et al, 1982) and sequenced by the dideoxy chain terminating method of Sanger et al, Proc. Natl. Acad. Sci USA 74, 5463-5467 (1977) using Sequenase (United States Biochemical Corp., Cleveland, OH).

8. Additional sequence of the 100 kD transferrin binding protein gene from gonococcal strain FA19.

Using the general methods of examples 6 and 7, a chromosomal Sau3AI fragment of approximately 1.0 kbp is identified. This fragment is cloned into the BamHI site of the vector pHSS6-GCU (Elkins et al. v. Bacteriol, 173, 3911-3913 (1991.) (The GCU designation indicates that a 10 bp sequence, known as the gonococcal uptake sequence, is included in the vector.) This sequence is known to mediate species-specific uptake of DNA into the gonococcus (Elkins et al., Id.). The host strain for this cloning was HB101. The resulting clone is known as pUNCH 403.

The insert in pUNCH 403 is sequenced in its entirety using double stranded templates prepared according to the method described by Kraft et al. in Biotechniques 6, 554-556 (1988). The sequence is determined by means of Sanger's dideoxy method using Sequenase ™(United States Biochemicals). The sequence of the Sau3AI fragment in pUNCH 403 is shown in Figure 2A. The sequence in Figure 2A from nucleotides 1-558 represent the overlap between pUNCH 401 and pUNCH 402 (Figure 1) and pUNCH 403 (Figure 2A). One open reading frame exists from nucleotide 659 through nucleotide 1. Thus, the codon starting at nucleotide 69 (on the strand complementary to that shown in Figure 2A) which would encode a methionine residue, is the 5' end of the gene.

9. Evidence of structure and function of the 100 kD transferrin binding protein.

To determine the effect of inactivation of the 100 kD transferrin binding protein gene, transposon insertions are isolated along the length of the insert in pUNCH403 according to the protocol described in Seifert et al. in Genetic Engineering, Principals and Methods, Setlow, J.K. and Holleander, A., eds., Plenum Press, N.Y., Vol. 8, pages 123-134. mTn3CAT transposons are inserted by shuttle mutagenesis in E. coli, and chloramphenicol

resistant transformants are then selected in FA19 to create mutants. mTn3CAT transposons are referred to by Seifert et al. as m-Tn3(Cm). Mutants are then scored for their ability to grow on transferrin as their sole iron source and their ability to express the 100 kD protein as assayed by Western blot. The results of that experiment are shown in Figure 3. Transposons at positions designated "I", 44, 37, and 24 ablate both expression of the 100 kD protein and its ability to grow on transferrin. The transposon at position "A", however, allowed some growth on transferrin and the expression of some detectable native length transferrin binding protein. These results confirm the hypothesis that the structural gene encoding the 100 kD protein begins at position 659 (see Figure 2A), since an insertion upstream of this point allows expression of the wild-type length protein. The fact that expression is not detected at wild-type levels in mutant "A" indicates that the region upstream of the putative start codon is important for regulation of the gene encoding the 100 kD protein.

10. <u>The construction and screening of meningococcal genomic library.</u>

The 95 kD meningococcal transferrin binding protein gene was cloned in three steps. In the first step, using

gonococcal antiserum, a 1.3 kb HincII/EcoRI fragment from a lambda Zap II library was identified (see Figure 4). The sequence of this fragment is given in Figure 5. The putative start codon is indicated by an arrow, which also shows the direction of transcription. The preceding ribosome binding site is underlined. The 1.3 kb fragment contains about 500 bp of the 95 kD protein structural gene. This clone hybridized to a single 5 kb ClaI fragment in the meningococcal strain FAM20 chromosome. A partial 5 kb ClaI library in the vector pHSS6-GCU (as described in example 8) was constructed, and a 5 kb ClaI fragment using the 1.3 kb fragment as a probe was cloned. Partial restriction mapping of this ClaI fragment suggested that this fragment does not encode the entire structural gene. Therefore, in step 3, a 3.5 kb clal/EcoRI fragment (generated from the 5 kb ClaI fragment obtained in step 2) was used as probe, resulting in the cloning of the adjacent HincII fragment from a lambda Zap II library (Stratagene). This HincII fragment is about 3.5 kb in size, and probably encodes the rest of the 95 kD protein structural gene. This 3.5 kb HincII fragment is sequenced by generating the unidirectional deletions using Exonucleases III and VII as described by E. Ozkaynak and S.D. Putney in Biotechniques 5, 770 (1987).

11. Evidence of structure and function of the 95 kD transferrin binding protein.

The 1.3 kb HincII/EcoRI fragment was used to mutagenize the meningococcal 95 kD protein gene. The same shuttle mutagenesis procedure described in example 9 was employed, except that, instead of mTn3CAT transposons, mTn3erm transposons were introduced into the 1.3 kb clone. mTn3erm transposons were made by modifying the mTn3CAT transposons described in example 9 so as to confer erythromycin resistance. This modification permits erythromycin resistant meningococcal transformants to be selected. These transformants were screened for their ability to grow on transferrin plates as described in example 9. Results of this mutagenesis experiments are detailed in Figure 6. While mTn3erm insertions 1 and 2 completely abolished the expression of the 95 kD protein and the ability of the clones to grown on transferrin plates, mTn3erm insertions 3 and 4 exhibited some growth on transferrin and showed some amount of 95 kD protein on Western blots. Based on the sequencing and mutagenesis data it appears that the mTn3erm insertions 1 and 2 are in the structural gene and promoter region, respectively, while insertions 3 and 4 seem to be in an upstream region that might be involved in the positive regulation of expression.

SUPPLEMENTAL REFERENCES

The invention as claimed is enabled in accordance with the specification and readily available references and starting materials. Nevertheless, the following cell lines have been deposited in the American Type Culture Collection, Bethesda, Maryland on July 16, 1990 in order to facilitate the making and using of the invention:

Meningococcal cell line FAM18 (Accession Number ATCC 55071)

Meningococcal cell line FAM20 (Accession Number ATCC 55072)

Gonococcal cell line FA19 (Accession Number ATCC 55073)

In addition, the following brochures containing useful protocols and information are available in the file history of this specification.

"Predigested Lambda Zap/Eco RI Cloning Kit Instruction Manual," Stratagene, La Jolla, California (November 20,1987);

"Gigapack Plus" (for packaging recombinant lambda phage), Stratagene, La Jolla, California (April 25, 1988);

"picoBlue Immunoscreening Kit" Instruction Manual," Stratagene, La Jolla, California (May 19, 1989); and

"Genius Nonradioactive DNA Labeling and Detection Kit," Boehringer Mannheim Biochemicals, Indianapolis, Indiana (January, 1989).

Claims

1. An iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis; and

wherein the protein is isolatable by means of a transferrin affinity column; and

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins.

2. The protein of claim 1 wherein the protein is found in Neisseria gonorrhoeae and has a molecular weight of 100 kD.

3. The protein of claim 1 wherein the protein is found in Neisseria meningitidis and has a molecular weight of 95 kD.

4. The protein of claim 1 wherein the protein is the Neisseria gonorrhoeae or Neisseria meningitidis transferrin receptor.

5. The protein of claim 1 wherein the protein is labeled.

6. Isolated antibodies raised against an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis; and

wherein the protein is isolatable by means of a transferrin affinity column; and

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane

protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins.

7. The antibodies of claim 6 wherein the antibodies are suitable for detecting the presence of Neisseria gonorrhoeae or Neisseria meningitidis in a sample.

8. The antibodies of claim 6 wherein the antibodies are suitable for treating a mammal infected with N. gonorrhoeae or N. meningitidis.

9. The antibodies of claim 6 wherein binding of the antibodies to the protein inhibits growth of Neisseria gonorrhoeae or Neisseria meningitidis.

10. The antibodies of claim 6 wherein the binding of the antibodies blocks transferrin receptor function of the protein.

11. The antibodies of claim 6 wherein the antibodies are monoclonal.

12. A vaccine composition comprising an effective amount of an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD;

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis, and functional analogs of such proteins; and

a pharmaceutically acceptable carrier.

13. The vaccine of claim 12 wherein the protein is found in Neisseria gonorrhoeae and has a molecular weight of 100 kD.

14. The vaccine of claim 12 wherein the protein is found in Neisseria meningitidis and has a molecular weight of 95 kD.

15. A method of immunizing a mammal against diseases caused by N. gonorrhoeae or N. meningitidis comprising the step of administering to the host a vaccine composition comprising an effective amount of an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane

protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD;

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis, and functional analogs of such proteins; and

a pharmaceutically acceptable carrier.

16. A method of treating a mammal suffering from diseases caused by N. gonorrhoeae or N. meningitidis, the method comprising administering to the mammal an effective amount of isolated antibodies raised against an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins.

17. A nucleotide sequence encoding an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(a) detergent;

(b) nitrocellulose/cellulose acetate paper; and

(c) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins and functional derivatives of such proteins.

18. A method for detecting the presence of antibodies specific for N. gonorrhoeae and N. meningitidis in a sample, the method comprising the steps of:

(a) incubating a protein according to claim 1 with a sample suspected of containing antibodies to an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(1) detergent;

(2) nitrocellulose/cellulose acetate paper; and

(3) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane

protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins;

(b) labeling the protein either before, during, or after the incubation of step (a); and

(c) detecting the labeled protein bound to an antibody in the sample.

19. A method for detecting the presence of N. gonorrhoeae or N. meningitidis in a sample comprising the steps of:

(a) incubating a sample suspected of containing N. gonorrhoeae or N. meningitidis with an antibody raised against an iron-regulated protein found in Neisseria gonorrhoeae or Neisseria meningitidis outer membranes, wherein the protein is substantially free of:

(1) detergent;

(2) nitrocellulose/cellulose acetate paper; and

(3) other iron-regulated proteins from Neisseria gonorrhoeae and Neisseria meningitidis;

wherein the protein is isolatable by means of a transferrin affinity column;

wherein the protein binds specifically to antisera raised against an iron-regulated outer membrane protein found in Neisseria gonorrhoeae having a molecular weight of approximately 100 kD; and

wherein the protein is important in transferrin receptor function in Neisseria gonorrhoeae or Neisseria meningitidis; and functional analogs of such proteins;

(b) labeling the antibody either before, during, or after the incubation of step (a); and

(c) detecting the presence of antibody bound to the protein.

FIGURE 1

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   1  AGCAAAGCCT CCGCACCGCC GATGCGCCCC GCCAGCGCGA TGGATTGGGT
  51  AAGCCCCCGG TTTTTGCCGG AATAGGCGGT TTTACTCTGA ATGCCCCACT
 101  GCCTGCCTTC CCCGATAACA TCGTCGGCGG TTTTGGTTTG AAATGCGACC
 151  GAGCCCGCCA ATGCGCCGCT GCCTTGTTCG ACCGAGTTTG AGCCTTTGCT
 201  GATTTCGACA GCCTTGACGT TCTCATACTC GATTTCATTG ATTGCGCCGC
 251  TGCTGCCCGC CGTCCTCGTC CCGCCCAATG CCGCCTGCGC GTGTAGGACT
 301  GTATTTGCGC CAAGCCGTCC ACCGTCAAGG AGACGCGGTT TTTGTCCATA
 351  CCGCGTATCG AGTAGCCCGA GCTTGCGCCG CGCCCCTGTT CGACGACGGC
 401  GATGCCGGGG TCGTAACGCG TCAGGTCGCG GATGTCGAGT ACCTGTTCCT
 451  TGCTGGAGGG TGTCGGCGGT TTTGACCAAT TTGCCCAAAC CGGTTACTTC
 501  GTTATCGCGG CGGGTTTTCT GTTTTTTGGC TTTTACCTGT ATGGTATCCA
 551  ACTGTTTT
```

FIGURE 2A

```
   1  AGCAAAGCCT CCGCACCGCC GATGCGCCCC GCCAGCGCGA TGGATTGGGT
  51  AAGCCCCCGG TTTTTGCCGG AATAGGCGGT TTTACTCTGA ATGCCCCACT
 101  GCCTGCCTTC CCCGATAACA TCGTCGGCGG TTTTGGTTTG AAATGCGACC
 151  GAGCCCGCCA ATGCGCCGCT GCCTTGTTCG ACCGAGTTTG AGCCTTTGCT
 201  GATTTCGACA GCCTTGACGT TCTCATACTC GATTTCATTG ATTGCGCCGC
 251  TGCTGCCCGC CGTCCTCGTC CCGCCCAATG CCGCCTGCGC GTGTAGGACT
 301  GTATTTGCGC CAAGCCGTCC ACCGTCAAGG AGACGCGGTT TTTGTCCATA
 351  CCGCGTATCG AGTAGCCCGA GCTTGCGCCG CGCCCCTGTT CGACGACGGC
 401  GATGCCGGGG TCGTAACGCG TCAGGTCGCG GATGTCGAGT ACCTGTTCCT
 451  TGCTGGAGGG TGTCGGCGGT TTTGACCAAT TTGCCCAAAC CGGTTACTTC
 501  GTTATCGCGG CGGGTTTTCT GTTTTTTGGC TTTTACCTGT ATGGTATCCA
 551  ACTGTTTTtc ctgtgcttgt ccggcttgca cattttctgc ataagcgggc
 601  agcgcagtca ttaaagacag gcataaaata tttaatcgga acaaatgttg
 651  ctgttgcata gtgtttccct aatcttcgct ttcagacgga atcggaagga
 701  acgatgccgt ctgaagcctt attctcgatt gttgttcggc agccgtgctt
 751  atttcacaag cttttggcgt ttcgcaccga atacgacagt tgcactgctt
 801  gctgaatttc cattgccgga ttcaactgtt gcatttttcg tttgttcatt
 851  gcccggatag gcaaaccatc cgcccaactc ttcggcgtta ggcccgtaaa
 901  aaccgccctg cacttcgg
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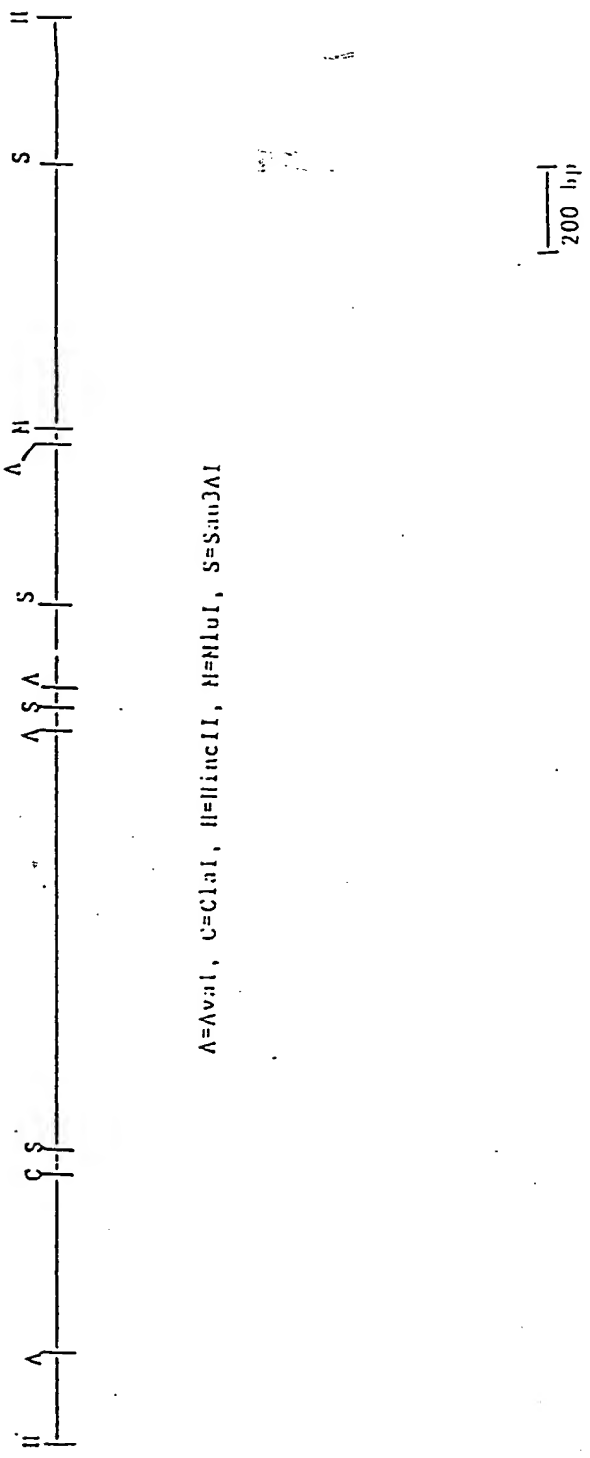


FIGURE 2B

FIGURE 3

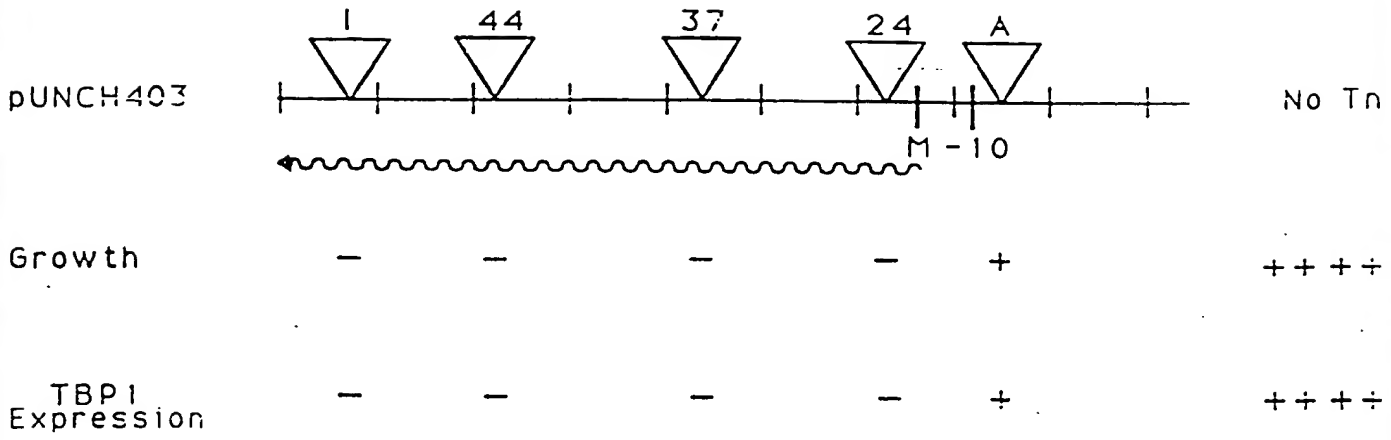


FIGURE 4: Strategy for cloning meningococcal transferin binding protein gene:

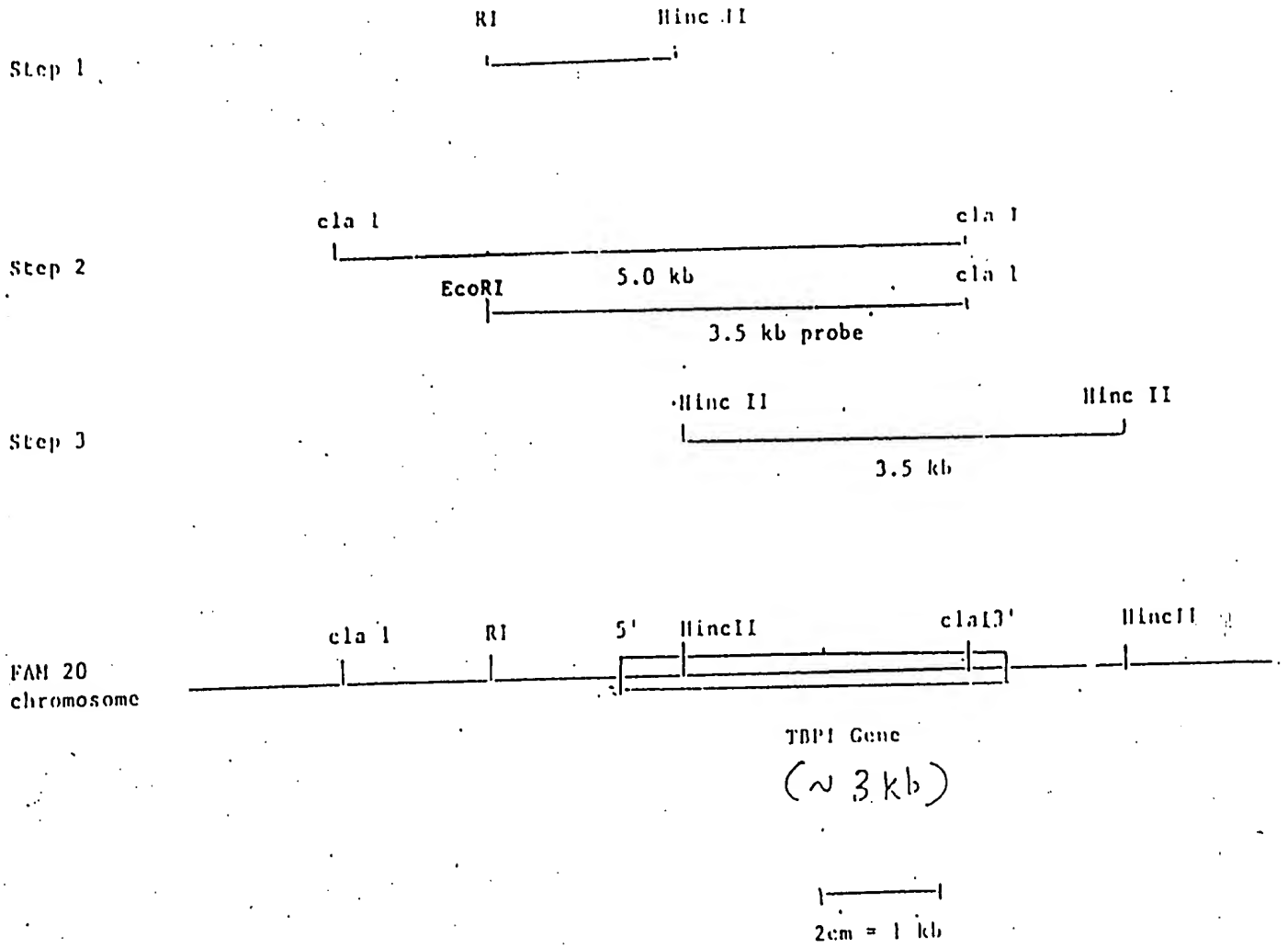


Figure 5: Sequence of 1.3 kb HincII/RI fragment (1192 bases)

AATTCCGACGGAGTGGAGCTTTCACTGCTGCCGTCTGAGGGCAATAAGGCGGCATTTCAGCACGAGATTGAGCAAAACGG

CGTGAAGGCAACGGTGTGTTGTTCCAACTTGGATTACATGAGTTTTGGGAAGCTGTCAAAAGAAAATAAAGACGATATGT

TCCTGCAAGGTGTCCGCACTCCTGTATCCGATGTGGCGGCAAGGACGGAGCAAACGCCAAATATCGCGGTACTTGGTACG

GATATATTGCCAACGGCACAAGCTGGAGCGCGAAGCCTCCAATCAGGAAGGTGGTAATAGGGCAGAGTTTGACGTGGATT

TTTCCACTAAAAAAATCAGTGGCACACTGACGGCAAAAGACCGTACGTCTCCTGCGTTTACTATTACTGCCATGATTAAG

GACAACGGTTTTTCAGGTGTGGCGAAAACCGGTGAAAACGGCTTTGCGCTGGATCCGCAAAATACCGGAAATTCCCACTA

TACGCATATTGAAGCCACTGTATCCGGCTGGTTTCTACGGCAAAAACGCCATCGAGATGGGCGGATTCGTTCTCATTTCC

GGGAAATGCACCAGAGGGAAAACAAGAAAAAGCATCGGTGGTATTCGGTGCGAAACGCCAACAGCTTGTGCAATAAGCAC

GGCTGCCGAACAATCGAGAATAAGGCTTCAGACGGCACGTTCCTTCCGATGCCGTCTGAAAGCGAAGATTAGGGAAACAT

CATGCAACAGCAACATTTGTTCCGATTAAATATTTTATGCCTGTCTTTAATGACCGCGCGCTGCCCGTTTATGCAGAAAA

TGTGCAAGCCGAACAAGCACAGGAAAAACAGTTGGATACCATACAGGTAAAAGCCAAAAAACAGAAAACCCGCCGCGATA

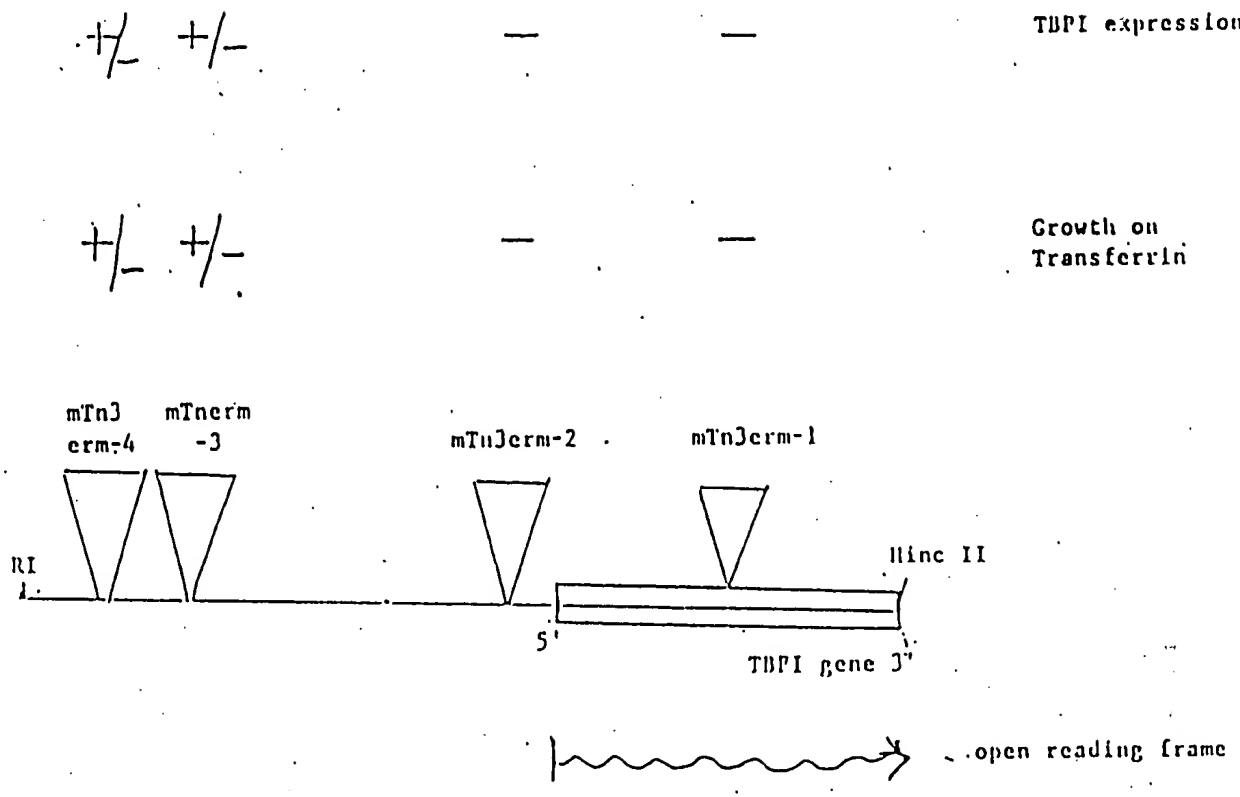
ACGAAGTAACCGGGCTGGGCAAGTTGGTCAAGTCTTCCGATACGCTAAGTAAAGAACAGGTTTTGAATATCCGAGACCTG

ACCCGTTATGATCCGGTATTGCCGTGGTCGAACAGGGTCGGGGCGCAAGTTCGGCTATTCAATACGCGGCATGGATAAAA

ACCGCGTTTCCTTAACGGTAGACGGCGTTTCGCAAATACAGTCCTACACCGCGCAGGCGGCATTGGGTGGGACGAGGACG

GCGGGTACGAGCGGCGCAATCAATGAAATCGAGTATGAAAACGTCAAGGCCGTTGAAATCAGCAAGGGTTCG

FIGURE 6: Transposon mutagenesis of 1.3 kb fragment



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06026

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.CL:530/350,403;424/92; 435/7.2; 530/387; 536/27
IPC(5): C07K 3/00; A61K 39/02; C12Q 1/00; C07H 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/350,403; 424/92; 435/7.2; 530/387, 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, MEDLINE, BIOSIS, PERSONAL TEXTS AND DATABASE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	PATHOGENIC NEISSERIAE. PROCEEDINGS OF THE FOURTH INTERNATIONAL SYMPOSIUM. issued 1985. West et al. "Iron Acquisition by the Pathogenic Neisseriae". pages 415-422. see page 420.	1-19
Y	MOLECULAR MICROBIOLOGY. Volume 2. No. 2. issued 1988. Schryvers et al.. "Identification and Characterization of the Transferrin Receptor from Neisseria Meningitidis". pages 281-288. see the Abstract and page 285.	1-19
Y	FEMS MICROBIOLOGY LETTERS. Volume 69. issued May 1990. Griffiths et al.. "Antigenic and Molecular Heterogeneity of the Transferrin-Binding Protein of Neisseria Meningitidis". pages 31-36. see the Abstract.	1-19

[10] Special categories of cited documents: [11]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 December 1991	10 JAN 1992

International Searching Authority	Signature of Authorized Officer
ISA/US	David R. Preston

Form PCT/ISA/210 (second sheet) (Rev. 11-87)